Biomolecular profiling of metastatic prostate cancer cells in bone marrow tissue using FTIR microspectroscopy: a pilot study

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Abstract Prostate cancer (CaP) cells preferentially metastasise to the bone marrow, a microenvironment that plays a substantial role in the sustenance and progression of the CaP tumour. Here we use a combination of FTIR microspectroscopy and histological stains to increase molecular specificity and probe the biochemistry of metastatic CaP cells in bone marrow tissue derived from a limited source of paraffin-embedded biopsies of different patients. This provides distinction between the following dominant metabolic processes driving the proliferation of the metastatic cells in each of these biopsies: glycerophospholipid synthesis from triacylglyceride, available from surrounding adipocytes, in specimen 1, through significantly high ($p \leq 0.05$) carbohydrate ($8.23 \pm 1.44 \text{ cm}^{-1}$), phosphate ($6.13 \pm$

1.5 cm^{-1}) and lipid hydrocarbon ($24.14 \pm 5.9 \text{ cm}^{-1}$) signals compared with the organ-confined CaP control (OC CaP), together with vacuolation of cell cytoplasm; glycolipid synthesis in specimen 2, through significantly high ($p \leq 0.05$) carbohydrate ($5.51 \pm 0.04 \text{ cm}^{-1}$) and high lipid hydrocarbon ($17.91 \pm 2.3 \text{ cm}^{-1}$) compared with OC CaP, together with positive diastase-digested periodic acid Schiff staining in the majority of metastatic CaP cells; glycolysis in specimen 3, though significantly high ($p \leq 0.05$) carbohydrate ($8.86 \pm 1.78 \text{ cm}^{-1}$) and significantly lower ($p \leq 0.05$) lipid hydrocarbon ($11.67 \pm 0.4 \text{ cm}^{-1}$) than OC CaP, together with negative diastase-digested periodic acid Schiff staining in the majority of metastatic CaP cells. Detailed understanding of the biochemistry underpinning the proliferation of tumour cells at metastatic sites may help towards refining chemotherapeutic treatment.

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Introduction

In the UK, prostate cancer (CaP) is the most commonly diagnosed cancer in men [1] and the second most common cause of cancer-related death of men [2]. It is well documented that circulating metastatic CaP cells in the peripheral blood possess a predilection for deposition into the bone marrow [3] and this phenomenon is directly responsible for considerable morbidity and mortality. The median survival following presentation with bone metastasis is approximately 18 months [4].

The bone marrow is a multicellular and highly metabolically active tissue. The presence of haematopoietic stem

cells in the bone marrow is responsible for the formation of red cells, osteoclasts and macrophages that are derived from the myeloid lineage haemopoietic cells, as well as mesenchymal cells that differentiate into oestoblasts, chondroblasts, fibroblasts and adipocytes [5]. Thus, the bone marrow is a fertile microenvironment, generating a vast array of cytokines and growth factors [6], which can stimulate and/or regulate the growth of infiltrating tumour cells [7]. Similarly, growth factors and cytokines released by CaP cells in the bone marrow can stimulate and/or regulate osteoblast and/or osteoclast cells at the site of metastases, leading to an imbalance in bone remodelling [8]. Therefore, cross-talk between stromal components and tumour cells is paramount for the survival and proliferation of CaP cells in the bone marrow.

Therapeutic regimes have been developed for interrupting tumour growth within the bone marrow, based on the knowledge of the interactions of tumour cells with bone marrow components. These include drugs such as bisphosphonates or bone morphogenic proteins that target osteolysis [9]. However, as a result of the complexity of the bone marrow microenvironment, these drugs possess only a retardation effect on CaP progression. Thus, there is a strong initiative towards a better understanding of the molecular mechanisms governing the interactions between tumour cells and bone so that key biochemical pathways can be inhibited to diminish the progression of tumour cells or the propensity for additional metastases [10].

In recent years Fourier transform infrared (FTIR) microspectroscopy has been applied to a variety of problems in biomedical science that include probing the biochemistry of abnormally proliferating [11] or malignant cells within biopsy tissue [12]. FTIR microspectroscopy is an optically based technique that can measure the transitions in vibrational modes (mainly stretching and bending) of the functional groups of biomolecular constituents within tissue, as a result of absorption and subsequent excitation by IR photons. The functional group vibrations are representative of cellular constituents such as lipids, proteins, carbohydrates and a variety of phosphorylated molecules. The IR spectrum derived from the tissue comprises of a series of peaks as a function of wavenumber, that depict the state of chemical bonding (intra- and inter-molecular hydrogen bonding, van der Waals interactions, steric factors and inductive or resonance effects) and provide an indication of the relative intensities of the abovementioned biomolecules within the analysed area of tissue and without the requirement for molecular tracers.

Previously, we have demonstrated using FTIR microspectroscopic imaging that metastatic CaP cells in bone marrow tissue, with close proximity to adipocyte-rich centres (storage cells for fat), give rise to higher intensity

lipid hydrocarbon signals relative to CaP cells further away [13]. Moreover, recent epidemiological and cellular biochemical investigations have demonstrated the complex associations between lipids and prostate tumour growth [13–16]. Since bone marrow is an adipocyte-rich environment, these studies are of particular relevance towards understanding the biomolecular mechanisms by which metastatic CaP cells proliferate within the skeletal tissue.

In the present study we use FTIR microspectroscopy to interrogate the biochemistry of metastatic CaP cells in primary bone marrow tissue sections that are derived from different patients and compare these biomolecular profiles to those obtained from high-grade prostate organ-confined CaP. We also demonstrate an increase in biomolecular specificity by comparing our FTIR data with histological data using the periodic acid Schiff stain and diastase control. This was carried out to highlight the lack of specificity in determining biomolecular classes using each technique independently (i.e. histological stains or FTIR microspectroscopy) and the advantages of combining these techniques to increase specificity for elucidating biomolecules present within the tissues. It is important to note that this manuscript is concerned with the use of IR spectroscopy to provide information regarding potential chemotherapeutic targets rather than demonstrating the use of this technology for routine histological assessment of CaP bone metastases biopsies, since this would not be a situation presented to the histopathology department.

Methods and materials

Primary tissue preparation

Five paraffin-embedded blocks of Gleason grade 5, CaP and four CaP bone metastases biopsies were obtained with informed consent from men with metastatic CaP (Genito-Urinary Cancer Research Group, CR-UK Paterson Institute). Of the four CaP bone metastases biopsies, two were derived from different patients (specimens 1 and 4 in the following text) and two were derived from the same patient (specimens 2 and 3 in the following text). These were sectioned at 10- μ m thickness and mounted onto BaF₂ plates. The section was then washed on an orbital mixer with CitrocLEAR for 20 min to remove the paraffin and then acetone for a further 20 min before being air-dried under ambient conditions. This process may remove tissue lipid molecules; however, our FTIR results reported in the following text, demonstrate that at least some of these lipids, glycerol-bound and free fatty acids, have been retained within the tissue as noted by the presence and differences in frequency of absorption for the lipid $\nu_s(\text{C=O})$

band (see [Results and Discussion](#)). Additionally, it is known that detergent-resistant lipid domains [17] are not perturbed by lipid leaching reagents and these lipids are retained with the tissue. Since these are located in the plasma membrane, they could protect the interior of the cell from further lipid removal. Finally, we assume that the dewaxing process, which is identical for all specimens, removes the same amount of lipid across the tissue section and between specimens so that the relative amounts of retained lipids between specimens are not significantly changed. In fact, we have previously reported an FTIR spectral map of unstained bone marrow tissue exhibiting infiltrating metastatic CaP cells [13], processed in an identical way as to tissues used in the present manuscript. This map demonstrates a lipid intensity distribution that is higher in CaP cells closer to adipocytes compared to those that are further away. This differential distribution of lipid intensity has been attributed to lipid uptake by CaP cells from adipocytes. If lipid leaching due to sample processing were different between locations on the same tissue section, then we would not expect to observe this association between CaP cells and adipocytes.

Targeted IR spectra of malignant prostate epithelial cells within the unstained tissue matrix involved the collection of a serial section(s), from the specimen block, which was stained using haematoxylin and eosin (H&E) and/or periodic acid Schiff (PAS) stain (with and without diastase pre-treatment). The anatomical features identified from the H&E section were used as landmarks for the positioning of the IR beam on the complementary unstained section. In the present study, areas for analysis were assigned by two histopathologists (J.H.S and J.R).

Adipocyte cell culture

Human preadipocyte cells, isolated from subcutaneous fat (Cambrex, UK), were cultured in preadipocyte growth medium (preadipocyte basal medium with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin) at 37 °C, 5% CO₂ for 7 days. Once the cells had reached 70% confluency the preadipocyte cells were subcultured onto 70% v/v ethanol-sterilised MirrIR slides. Induction of adipogenesis was carried out by replacing the preadipocyte growth medium with adipocyte differentiation medium (preadipocyte growth medium with 5 µg/mL insulin, 0.5 µM dexamethasone, 100 µM indomethacin and 250 µM isobutylmethylxanthine) and incubation at 37 °C, 5% CO₂ for 10 days. Differentiated adipocyte cells were fixed in 4% formalin and briefly washed in deionised water for 3 s and air-dried. The effects of this fixation protocol on localisations of IR biomolecular signals from FTIR images of single cells have been reported in ref. [18].

FTIR microspectroscopy of tissue

FTIR spectra of CaP cells in bone marrow as well as Gleason graded primary prostate tissues were collected in transmission mode using a Nicolet Magna system 550 spectrometer equipped with a liquid-nitrogen-cooled MCT/A detector and a KBr beam splitter. An aperture size of 60 µm×60 µm was used to collect IR spectra, specifically from the CaP tissue. FTIR spectra represent an average of 512 scans in the wavenumber range 750–4,000 cm⁻¹ with a resolution of 4 cm⁻¹. Background scans were obtained from a region of no sample and ratioed against the sample spectrum.

FTIR microspectroscopy of reference lipids

Arachidonic acid–5,8,11,14 and triacylglyceride mix (containing triacetin (C2:0), tributyrin (C4:0), tricaproin (C6:0), tricaprylin (C8:0), tricaprin (C10:0)) were analysed in reflection mode by FTIR (at 4 cm⁻¹ spectral resolution with 512 co-added scans) after smearing small amounts onto MirrIR slides. Both chemicals were obtained from Sigma–Aldrich, UK.

SR-FTIR microspectroscopy of an adipocyte cell

FTIR spectra of a formalin-fixed adipocyte cell were collected at the Daresbury Laboratory synchrotron radiation (SR) source using beamline 11.1. For this, a Nicolet Nexus FTIR spectrometer coupled to a Continuum IR microscope, equipped with a liquid-nitrogen-cooled MCT/A detector and a ×32 Cassegrain objective was used. SR-FTIR spectra

Fig. 1 Normal bone marrow tissue exhibiting a densely packed population of numerous cell types (see text). Scale bar=50 µm

were collected in reflection mode at 4 cm^{-1} resolution with 128 co-added scans, using a $7\text{ }\mu\text{m} \times 7\text{ }\mu\text{m}$ sampling aperture.

Data processing

Data processing was carried out using the OMNIC v.5.1a software, which included the Atl μ s video capturing program. This software was used for linear baseline correction across the whole spectral range, $4,000\text{--}750\text{ cm}^{-1}$. A 2-point linear baseline was applied between the higher-frequency side of the amide I peak (ca. $1,720\text{ cm}^{-1}$) to the higher-frequency side of the phosphate monoester peak (ca. 980 cm^{-1}). The spectra were then normalized to the amide I (ca. $1,650\text{ cm}^{-1}$) peak intensity. The OMNIC-Atl μ s software also performed second-derivative calculation of absorption spectra. The Microcal Origin v.4.1 program performed deconvolution to resolve the underlying and overlapping peaks present as a composite of a broad peak.

Statistics

The non-parametric, Mann–Whitney U two independent samples test was used to determine significant differences in the peak area intensities of biomolecular signals between prostate organ-confined CaP and bone metastatic CaP.

Results and discussion

Figure 1 shows the appearance of a normal bone marrow tissue section. The normal bone marrow contains a densely packed population of different cell types that include adipocytes, haemopoietic cells and mesenchymal cells. Table 1 shows the H&E photomicrographs of bone marrow tissue sections with infiltrating metastatic CaP cells, derived from four biopsies. These H&E sections demonstrate replacement of normal bone marrow by metastatic CaP cells and fibrocollagenous stroma. Unstained tissue sections cut adjacent to each of these H&E tissue specimens were used for analysis by FTIR microspectroscopy.

Table 1 H&E and PAS/diastase-treated PAS-stained photomicrographs of bone marrow tissue exhibiting metastatic CaP

Specimen Number	H&E*		PAS	Diastase Digested PAS
	Low Magnification	High Magnification		
1
2 ^a
3 ^a
4

* Green asterisk designates an example of an adipocyte cell in the tissue section. Scale bar= $50\text{ }\mu\text{m}$, except for low magnification H&E where scale bar= $100\text{ }\mu\text{m}$; a. These biopsies were derived from the same patient.

The data obtained from 12 locations upon each section are presented in Fig. 2. The spectra were used to determine the biomolecular intensities of the carbohydrate, phosphate and lipid hydrocarbon signals for each of these tissue specimens (Fig. 3b). The methods by which these biomolecular signals were obtained are illustrated in Fig. 3a. The lipid hydrocarbon (peak area 1; sum of the $\nu_{as/s}(\text{CH}_2)$ and $\nu_{as/s}(\text{CH}_3)$ bands) and phosphate (peak area 2; $\nu_{as}(\text{PO}_2)$) peak area signals were obtained by baseline integration of the band regions $3,000\text{--}2,800\text{ cm}^{-1}$ and $1,290\text{--}1,180\text{ cm}^{-1}$, respectively (Fig. 3ai); whereas, the carbohydrate signal was acquired through deconvolution of the broad spectral envelope between 950 and $1,183\text{ cm}^{-1}$ (Fig. 3aiii) [19, 20]. The carbohydrate signal (sum total of peak areas 3–7, corresponding to the $\nu_s(\text{C-O})$ at $1,023\text{ cm}^{-1}$, $\nu_s(\text{C-O-C})$ at $1,041\text{ cm}^{-1}$ and $1,055\text{ cm}^{-1}$ and $\nu_s(\text{C-O})$ coupled to the $\delta(\text{C-O-H})$ vibrations at $1,173$ and $1,154\text{ cm}^{-1}$ [19]) was acquired through deconvolution of the broad spectral envelope between 950 and $1,183\text{ cm}^{-1}$ (Fig. 3aiii). The number of Gaussian peaks used in the deconvolution process was determined by calculating the second-derivative spectrum for this band region (Fig. 3aii), which accurately identifies the centres of shoulders under the broad peak (black arrows in Fig. 3aii). Note that the relative intensity and band shapes of peaks are lost as a result of differentiation. For ease of visualisation and to permit a comparison with the original absorption spectral profile, the second-derivative spectrum was multiplied by (-1) . The

number of peaks elucidated by this method (10 peaks), for this spectral region, is supported by the work of Petibois et al. [21] who used free deconvolution alone to initially determine the number of absorption peaks within the same envelope for blood plasma. We followed a procedure similar to Petibois et al. whereby we used free deconvolution to initially fit 10 peaks (as determined by the second-derivative spectrum) into the $950\text{--}1,183\text{ cm}^{-1}$ spectral envelope. The peak widths and frequency at which the deconvoluted peaks absorb and absorption line shapes (Gaussian) were then fixed during Chi-Square iterative fitting. The biomolecular intensities derived from FTIR spectra of prostate organ-confined (OC) Gleason grade 5, CaP biopsies were used as a control in Fig. 3b. For the OC CaP specimen, the values in Fig. 3b represent the mean of 40 locations sampled by FTIR microspectroscopy from 5 biopsies (each biopsy derived from a different patient).

In the following text, we tabulate the histological data alongside the IR biospectroscopic data to determine the possible biochemical processes driving the proliferation of metastatic CaP cells in each of the four bone metastases tissue specimens and compare the IR data with those obtained from Gleason grade 5, organ-confined (OC) CaP cells. Since Gleason grade 5, OC CaP cells have the highest probability for distant metastasis, this control provides a baseline measurement of the biochemistry in potentially metastatic CaP cells within a non-hemopoietic microenvironment.

Fig. 2 Mean FTIR spectra of spectra collected from different metastatic CaP locations in 4 specimens of bone marrow tissue. The standard deviation ($\pm$) of absorption values is indicated in grey for each wavenumber

Fig. 3 **a** *i* Peak area by integration to determine the lipid hydrocarbon (peak area 1) and phosphate intensities (peak area 2). *ii* Second-derivative spectrum ($\times -1$) of the absorption spectrum was used to determine the position and number of peaks (black arrows) underlying the spectral envelope between 950 and 1,183 cm^{-1} , from which the *iii* carbohydrate intensity was determined by the sum of deconvoluted peak areas 3–7. **b** Histograms showing the relative intensities of *i* carbohydrate, *ii* phosphate and *iii* lipid hydrocarbon signals from CaP bone metastases tissue specimens (1–4) and organ-confined CaP (OC CaP; control); $*p < 0.05$ (compared to signal intensities of the organ-confined CaP control); standard deviation ($\pm$) bars are shown

Glycerophospholipid synthesis in bone marrow metastatic CaP cells

The H&E photomicrographs show that the cytoplasm of the CaP cells in specimen 1 (Table 1) is vacuolated (large pale

appearance), which can indicate large accumulations of glycogen, lipids or mucopolysaccharides. As a preliminary step to identify which of these biomolecules has caused vacuolation of cell cytoplasm in specimen 1, a periodic acid Schiff (PAS)-stained section was acquired. PAS stains

glycogen as well as glycoproteins, glycolipids and mucopolysaccharides with a magenta colouration [22]. A number of CaP cells in specimen 1 revealed strong positive staining for PAS, whilst others showed pale or negative staining (see Table 1). A diastase-digested PAS-stained section of specimen 1 is also shown in Table 1. Diastase is an enzyme used to remove glycogen and is routinely used in histological practices to confirm the presence of glycogen as PAS lacks specificity to this biomolecule. The diastase-treated PAS-stained section of specimen 1, reveals negative staining for PAS, indicating the presence of glycogen in the metastatic CaP cells of the PAS/H&E-stained section of specimen 1. Interestingly, the carbohydrate signals are significantly higher in specimen 1 (Fig. 3bi) compared with the OC CaP control, which might partly be due to the storage of glycogen in some of the CaP cells. The CaP cells

of specimen 1 also display significantly higher lipid hydrocarbon signal (Fig. 3biii) compared to the OC CaP control and represents the highest lipid hydrocarbon value (24.1 cm^{-1}) of all CaP bone metastases specimens studied. This high lipid hydrocarbon signal is accompanied by the presence of a peak at $1,738 \text{ cm}^{-1}$, which corresponds to the ester $\nu_s(\text{C}=\text{O})$ of lipids [20]. Moreover, the lipid ester $\nu_s(\text{C}=\text{O})$ peak is only observed in the two specimens that give rise to the two highest lipid hydrocarbon signals (specimens 1 and 2, Fig. 3biii) and was not observed in IR spectra taken from OC CaP cells [12].

The wavenumber at which the intensity maximum of the lipid ester $\nu_s(\text{C}=\text{O})$ peak absorbs can determine the presence of free or glycerol-bound lipids. This is demonstrated in Fig. 4a, which shows the neat FTIR spectrum of a glycerol-bound lipid, triacylglycerol (TAG) and the free

Fig. 4 a Molecular structure of triacylglyceride (TAG) (R_{1-3} are C2–C10 saturated hydrocarbon chains) and a typical fatty acid, arachidonic acid (AA) (R is the polyunsaturated C18 hydrocarbon chain) with their associated IR spectrum. **b** Optical image of a formalin-fixed adipocyte with a prominent lipid store. The inset shows a typical adipocyte in growth medium with well-structured lipid micelles. The white square in the centre of the lipid store in the fixed adipocyte cell represents the location at which the raw SR-FTIR spectrum was acquired

fatty acid, arachidonic acid (AA). We find that the lipid ester $\nu_s(\text{C}=\text{O})$ peak appears at higher wavenumber ($1,741\text{ cm}^{-1}$) in the FTIR spectrum of TAG compared with that in the spectrum of AA ($1,714\text{ cm}^{-1}$). The lipid ester $\nu_s(\text{C}=\text{O})$ peak in spectra of metastatic CaP cells of specimen 1 (Fig. 2) appears at $1,738\text{ cm}^{-1}$ and is closer to the value of the reference TAG spectrum than to the AA spectrum (Fig. 4a). Note, in TAG samples of human origin, a large proportion of the fatty acyl chains comprise longer chain lengths than used in the TAG standard in the present study. However, the length (or the extent of unsaturation) of the fatty acyl chains of the TAG standard does not make any significant difference to the frequency of the $\nu_s(\text{C}=\text{O})$ peak for the purposes of using it as a marker to differentiate fatty acids from TAG in our bone metastases CaP spectra. This is supported by the synchrotron radiation (SR)-based FTIR microspectroscopic analysis of the intercellular lipid store of a formalin-fixed adipocyte (Fig. 4b), which is known to store lipids (of various chain lengths that include the longer ($\geq \text{C}10$) acyl chains and number of double bonds) within its cytoplasm as TAG. The optical image in Fig. 4b shows that the lipid deposit (dark grey mass) occupies a large proportion of its cell body. SR-FTIR microspectroscopy enables an FTIR spectrum to be acquired at subcellular spatial resolution using an apertured beam size of $7\text{ }\mu\text{m} \times 7\text{ }\mu\text{m}$ with good signal to noise. The SR-FTIR spectrum of the cytoplasmic lipid store shows a lipid ester $\nu_s(\text{C}=\text{O})$ peak at higher wavenumber ($1,744\text{ cm}^{-1}$), which confirms that the lipids are present as TAG molecules. Thus, chain length or the extent of saturation do not affect our assignment of the $\nu_s(\text{C}=\text{O})$ peak as TAG at these higher frequencies that are well separated from the $\nu_s(\text{C}=\text{O})$ peak of fatty acids (that appear at frequencies $\leq 1,725\text{ cm}^{-1}$, discussed below). In addition, the presence of the $\nu_s(\text{C}=\text{O})$ peak at $1,744\text{ cm}^{-1}$ and the prominent $1,170\text{ cm}^{-1}$ peak corresponding to the $\delta(\text{C}-\text{O}-\text{H})$ vibration of glycerol in the SR-FTIR spectrum of the TAG deposit of the adipocyte (Fig. 4b), confirms that formalin-fixation (the primary method by which the tissue specimens of bone metastases specimens are preserved) maintains the chemical structural information of TAG.

The presence of high lipid intensities in FTIR spectra of CaP cells of specimen 1 may explain the negative staining of PAS in a proportion of these cells, since vacuolation of cell cytoplasm can result from lipid uptake [16]. This accumulation of lipids in metastatic CaP cells of specimen 1 could arise from the translocation and uptake of lipids from the nearby adipocyte cells, present in this section (see Table 1; the large vacuolated spaces identified by the green asterisk in the H&E-stained tissue of specimen 1 are adipocyte cells) [13]. The mean phosphate intensity for specimen 1 provides the highest value (6.1 cm^{-1}) of all bone metastases specimens; coupled with the intense lipid

hydrocarbon signal, this could suggest the presence of a higher proportion of glycerophospholipids within some of the CaP cells sampled by FTIR microspectroscopy. Figure 5a demonstrates the relationship between the lipid hydrocarbon and phosphate signals, through peak area values for each of these biomolecules for each FTIR spectrum acquired from the different CaP locations within specimen 1. In this figure we observe that a decrease in lipid hydrocarbon signal, obtained from each of these locations (1–12), is accompanied by an overall fall in phosphate signal, which can support the view that these signals represent synthesised glycerophospholipids. Note, the PAS stain cannot show positive staining for glycerophospholipids or TAG as these biomolecules do not contain the 1,2-glycol grouping with free $-\text{OH}$ groups, necessary for its positive reaction [22]. Thus, the negative PAS stain in CaP cells of specimen 1 (Table 1) does support the interpretation of the FTIR data presented above with respect to the presence of glycerophospholipid or TAG, rather than glycolipids that could potentially overlap with lipid hydrocarbon or lipid ester $\nu_s(\text{C}=\text{O})$ signals. Figure 5b shows that the intensity of the lipid ester $\nu_s(\text{C}=\text{O})$ peak at $1,738\text{ cm}^{-1}$ is greater than the intensity of this vibrational mode at lower wavenumber ($1,717\text{ cm}^{-1}$) in the majority of spectra collected from specimen 1 (locations 1–9). This supports the view that a greater proportion of glycerol-bound lipids (either as glycerophospholipids or TAG), compared with free fatty acids, are present in the majority of CaP cells sampled from different locations in specimen 1. The assignment of the $\nu_s(\text{C}=\text{O})$ peak at $1,717\text{ cm}^{-1}$ to free fatty acids is based on its absorption frequency that lies in between the spectral range $1,725\text{--}1,690\text{ cm}^{-1}$. This spectral range corresponds to reports by Gomez-Fernandez et al. [23] and Chalmers et al. [20] who have reported that the frequency of the free fatty acid $\nu_s(\text{C}=\text{O})$ peak occurs at these frequencies. In addition, the fatty acid standard spectrum presented in Fig. 4a shows absorption of the $\nu(\text{C}=\text{O})$ peak at $1,714\text{ cm}^{-1}$, which is a value also between $1,725$ and $1,690\text{ cm}^{-1}$ and is close to the $\nu(\text{C}=\text{O})$ peak at $1,717\text{ cm}^{-1}$ in the IR spectra from tissue spectra of specimen 1 (Fig. 5b). The differences in the position of the $\nu(\text{C}=\text{O})$ peak lies in the strength of the hydrogen bond with the COOH moiety.

We find no trend between the carbohydrate signal with lipid hydrocarbon signal for locations 1–12 (Fig. 5a), which one might expect if glycerol-bound lipids were present, since the glycerol moiety absorbs strongly at $1,170\text{ cm}^{-1}$ (see TAG reference spectrum in Fig. 4a and adipocyte lipid store spectrum in Fig. 4b). This irregularity is likely to be due to spectral overlap between carbohydrate signals arising from glycogen (as determined by comparison of the PAS section with the diastase-treated PAS-stained section for specimen 1) with those from the glycerol moiety of lipids.

Fig. 5 **a** Peak area intensities of the lipid hydrocarbon, phosphate and carbohydrate signals from FTIR spectra, each collected from random locations (1–12) in specimen 1. **b** Stacked FTIR spectra showing the absorption intensities of the lipid ester $\nu_s(\text{C}=\text{O})$ peak that depicts the relative proportions of glycerol-bound and free fatty acid forms of lipids in CaP cells from each location sampled in specimen 1. **c** Stacked FTIR spectra showing the difference in the value of the $\nu_{as}(\text{CH}_2)$: $\nu_{as}(\text{CH}_3)$ of lipid acyl chains, which demonstrate the length of lipid hydrocarbon chains in CaP cells from each location sampled in specimen 1

It is interesting to find that the decrease in lipid hydrocarbon signal between locations 1–12 in specimen 1 (Fig. 5a) is accompanied by a decrease in the ratio of the lipid ester $\nu_s(\text{C}=\text{O})$ peak of glycerol-bound:free fatty acids (Fig. 5b) and lipid $\nu_{as}(\text{CH}_2)$: $\nu_{as}(\text{CH}_3)$ of lipid acyl chains

(Fig. 5c). Together, these data can indicate the metabolism of longer hydrocarbon chains to shorter-chain free fatty acids as substrates for glycerophospholipids synthesis. Thus, we can group CaP cells from different locations in specimen 1, as characterised by their FTIR spectra, into

different biochemical categories: (a) CaP cells with higher proportion of synthesised glycerophospholipids (locations 1 and 2), (b) CaP cells with a higher proportion of stored lipid (locations 3–9) and (c) CaP cells with a higher proportion of shorter-chain free fatty acids (locations 10–12).

It is well known that the synthesis of membrane phospholipids is important for the development of new organelles in proliferating cells. Pizer et al. [24] have reported that inhibition of fatty acid synthase (FAS; an enzyme involved in the synthesis of fatty acids) decreases DNA synthesis, resulting in cells that do not pass the S-phase of their cell cycle. These authors suggest that the link between FAS and DNA replication may be the result of the need for the synthesis of membrane phospholipids, which is necessary for the development of new organelles when cells divide. Thus, the results of this present study of specimen 1 support the suggestion put forward by Pizer et al. [24] in that fatty acids play a crucial role in cellular proliferation.

Glycolipid synthesis and glycogen storage in bone marrow metastatic CaP cells

CaP bone metastases specimens 2 and 3 were obtained from different biopsies of the same patient. The H&E-stained sections from both of these specimens demonstrate that the CaP cells are similar in size and shape (Table 1) and form nests with intervening fibrocollagenous stroma. Adipocytes were observed in both tissue sections, although predominately distributed along the margins of the tumour tissue. The diastase-treated PAS-stained sections for both specimens demonstrate positive staining in some CaP cells, but, negative staining in others (Table 1) and reflects the heterogeneity in biochemistry across the tumour cells of these specimens. FTIR microspectroscopic sampling of these tissues reveals very different biomolecular intensity profiles for the carbohydrate, phosphate and lipid hydrocarbon signals (Fig. 3bi–iii).

The second highest mean lipid hydrocarbon signal is obtained from FTIR spectra of specimen 2 (Fig. 3biii) and is significantly greater than the lipid hydrocarbon signal of OC CaP. Since, the diastase-treated PAS-stained section of this specimen shows positive staining in some CaP cells (Table 1), this histochemical feature together with the high lipid hydrocarbon signal, suggests the presence of glycolipids or mucopolysaccharides. However, a vibrational mode that discriminates between glycolipid and mucopolysaccharide is the lipid ester $\nu_s(\text{C}=\text{O})$ peak at $1,738\text{ cm}^{-1}$, which is observed in the spectra of specimen 2 (Fig. 2) and indicates the presence of glycolipid. The lipid substrate for these glycolipids may also be derived from adipocyte cells observed near to the tumour tissue (Table 1, specimen 2; green asterisk designates adipocyte cell). Changes in the metabolism, organization and

composition of glycolipids such as glycosphingolipids are reported to be some of the most common changes associated with neoplastic transformation [25].

The carbohydrate intensity from CaP cells of specimen 3 is significantly greater than OC CaP (Fig. 3bi). This increase in carbohydrate intensity may be due to the CaP cells within specimen 3, which demonstrated negative diastase–PAS staining, suggesting the presence of intercellular stored glycogen. The phosphate signal for specimen 3 gives rise to the lowest mean value of all bone metastases specimens and is not significantly different from the phosphate value of the OC CaP control (Fig. 3bii). Since, phosphate moieties can arise in the form of energy-releasing molecules such as adenosine triphosphate (ATP) for the phosphorylation of proteins, the low phosphate signal provides supporting evidence for the lower metabolic activity in these CaP cells. The significantly lower lipid hydrocarbon signal compared with OC CaP and absence of the lipid ester $\nu_s(\text{C}=\text{O})$ peak confirms that we are measuring a greater proportion of diastase–PAS negative cells in specimen 3.

Association of biomolecular profiles to differentiation patterns of bone marrow metastatic CaP cells

The H&E photomicrographs of CaP cells of specimen 4 show high nucleus:cytoplasm ratio (Table 1) with a distinct cribriform pattern of growth, unlike the larger CaP cells that formed aggregated clusters/nests in specimens 1–3. The low lipid hydrocarbon signal in specimen 4, which was significantly lower than that found in OC CaP, may be associated with low lipid uptake or low production of lipid-containing biomolecules. Accordingly, the lipid ester $\nu_s(\text{C}=\text{O})$ peak was absent from all spectra recorded from specimen 4.

The PAS- or diastase–PAS-stained sections of specimen 4 did not show strong magenta staining. Negative staining may be due to artefactual staining efficiency as a result of the stain not reaching certain areas of the tissue. Staining artefact is not present in specimens 1–3, since cells that are PAS negative are surrounded by or in close proximity to stromal compartments, or other cells, that stain PAS positive (see photomicrographs in Table 1). However, specimen 4 may suffer from artefactual staining efficiency. This limitation of histology is an important reason for carrying out FTIR spectroscopy on tissue sections, since it provides additional information about the biochemistry of the cells that can assist the pathologist in determining whether variable staining is artefactual or biochemical.

Although the carbohydrate signal for specimen 4 was significantly greater than OC CaP, the carbohydrate value was low (relative to specimens 1 and 3) and may support the absence of strong positive staining in all CaP cells of

specimen 4. The low carbohydrate value could be due to a combination of a high rate of metabolism in the proliferating CaP cells and the lower capacity for its storage due to the low cytoplasm:nucleus ratio.

We postulate that these histological differentiation patterns and cytological differences exhibited by specimen 4, when compared to the other specimens (1–3), might result from differences in its interaction with the bone marrow microenvironment. It has been reported by Tokuda et al. [16] that adipocyte cells can influence the differentiation patterns of PC-3 cells (prostate cancer cells derived from bone metastases), *in vitro*. Since our FTIR data suggests significantly greater lipid accumulations in CaP cells of bone metastases biopsies from two patients, compared with prostate organ-confined CaP cells, the differences in differentiation patterns between specimens 4 (cribriform growth) and 1–3 (nests) could arise due to adipocyte interactions. The importance of adipocyte–CaP cell interactions is further supported by Brown et al. [13] who have reported that invasion of PC-3 cells towards human bone marrow stroma (BMS), *in vitro*, was significantly reduced when this assay was repeated with BMS tissue grown without hydrocortisone, which prevents the formation of adipocytes.

Conclusions

In summary, we have demonstrated that metastatic CaP cells in bone marrow, derived from different biopsies, possess distinct chemical profiles that may be interrogated by coupling FTIR microspectroscopic analysis with histological parameters. For one specimen, our data suggests that triacylglyceride (TAG) may have been incorporated into the CaP cells through the translocation of TAG from locally situated adipocytes within the tissue. Through the process of enzymatic breakdown and lipid biosynthesis, we postulate that a use of this TAG is for the generation of glycerophospholipids. For two other CaP bone metastases specimens, which were derived from the same patient, FTIR microspectroscopy highlights further important biochemical pathways (glycolipid synthesis and glycolysis) used to drive the proliferation of CaP cells within different regions of the tumour. With further research, biospectroscopic examination of tissue by FTIR microspectroscopy may play an important role, in combination with other methods of biomolecular characterisation such as histological staining reported here, to provide the basis for determining different chemotherapeutic targets for patients presenting with CaP metastases to the bone marrow. In addition, this study demonstrates the significance of FTIR technology to provide structural information as well as relative quantifications of a wide range of biomolecular

domains, economically, on a limited source of CaP bone metastases biopsies, which are not usually preformed.

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